Section 4)

Attachment no. 7

LSR-RTC S.P.A.:

FDP: Micronucleus test. BF file,

1989

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.P.A.

(1)

MICRONUCLEUS TEST

Test Substance: Fruttosio-1,6-difosfato

LSR-RTC Report No.: 003004-M-04688

FINAL REPORT

Seen and approved by:

A. Nunziata Pharm.D., Chem.D.
Responsible to Ministry of
Health for Experimentation

R.K. Haroz Ph.D.
Managing Director

LSR-RTC Report No.: 003004-M-04688

MICRONUCLEUS TEST

TEST SUBSTANCE: Fruttosio-1,6-difosfato

FINAL REPORT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted and the results obtained, in the performance of this study. The aspects of the study conducted by Life Science Research - Roma Toxicology Centre were performed essentially in accordance with:

A. "Good Laboratory practice Regulations" of the U.S. Food and Drug Administration, 21 CFR Part 58, 22-December-1978 and sections revised in Fed. Reg. 4-September-1987.

B. "Principles of Good Laboratory Practice relating to the conduct of Nonclinical Laboratory Studies" OECD Guidelines for the testing of Chemicals, Annex 2, (81) 30 (Final) 1981.

C. "Applicazione dei principi di buone pratiche di laboratorio sulle sostanze chimiche e criteri per il rilascio delle autorizzazioni previste dal decreto del Presidente della Repubblica n.927/81, art.6." Rome, Italy, D.M. No.76 Gazzetta Ufficiale del 27 Agosto 1986.

Assunta Dello Russo — 9 Marzo 1989
A. Dello Russo — Date
(Microscope slide scorer)

P. Mosesso — 9 Marzo 1989
P. Mosesso Bs.D. — Date
(Study Director)

8. Mai 89
R. Forster M.A.(Cantab) Ph.D. — Date
(Head of Genetic Toxicology)

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NINZIATA)

LSR-RTC Report No.: 003004-M-04688

Q.A. STATEMENT

Quality Assurance Inspections
(Day Month Year)

	Inspection	Report to Study Director	Report to Head of Responsible Department	Report to Company Management
PROTOCOL				
Inspection of protocol was made in accordance with LSR-RTC Standard Operating Procedure QAU/010.	17.06.88	20.06.88	20.06.88	26.07.88
DATA				
Inspection of data generated on this type of study was made in accordance with LSR-RTC Standard Operating Procedure QAU/030.	15.11.88 21.11.88 27.01.89	- - -	16.11.88 23.11.88 27.01.89	12.01.89 12.01.89 -
PROCEDURES				
Inspection of Procedures on this type of study was made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	17.11.88	23.11.88	23.11.88	12.01.89
Other routine procedures used in this type of and facilities were inspected regularly and reports were made in accordance with LSR-RTC Standard Operating Procedure QAU/020.	14.09.88 29.09.88 11.10.88 14.10.88 18.10.88 02.11.88 03.11.88 03.11.88 08.11.88 09.11.88 11.11.88 31.01.89	- - - - - - - - - - - -	23.09.88 04.10.88 28.10.88 21.10.88 21.10.88 15.12.88 03.11.88 14.12.88 11.11.88 11.11.88 15.12.88 02.02.89	10.11.88 10.11.88 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89 12.01.89

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This report has been reviewed by the LSR-RTC Quality Assurance Unit employing methods laid down in LSR-RTC Standard Operating Procedure QAU/040. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

This review was completed on: 8.03.89

V. Sforza, B.Sc.
(Quality Assurance Manager)

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(Dr. ALFREDO NUNZIATA)

CONTENTS

SUMMARY

1.1 The ability of fruttosio-1,6-difosfato to cause chromosomal damage in vivo was investigated in a micronucleus test. Dose-levels were selected as 80% and 40% of the oral LD50 value. The high and low dose-levels were calculated as 700 and 350 mg/kg respectively.

1.2 Swiss CD-1 mice were dosed once intravenously, with vehicle only (sterile distilled water), fruttosio-1,6-difosfato (at the selected dose-levels) or with the positive control substance Mitomycin-C (5.00 mg/kg) or Busulfan (80.0 mg/kg). Each treatment group consisted of five male and five female animals: groups were sacrificed at three sampling times which were 24, 48 and 72 hours after treatment. Bone-marrow smear slides were made and stained with May-Gruenwald and Giemsa stains. Where the toxicity of the test compound did not inhibit cell proliferation, approximately 1000 polychromatic erythrocytes (PCE's) per animal were examined for the presence of micronuclei. The slides were coded prior to scoring. The results obtained at each sampling time were subjected to statistical analysis using a modified chi-squared test.

1.3 No marked increases in the incidence of micronucleated PCE's (compared with the vehicle control values) were observed at any dose-level within any sampling time in the fruttosio-1,6-difosfato treatment groups.

Slight increases in the ratio of NCE's to PCE's were observed following fruttosio-1,6-difosfato treatment at the 24 hour sampling time, suggesting that the test substance was mildly inhibitory to erythropoietic cell division.

Statistically significant increases in the incidence of micronucleated PCE's over control values were seen in the positive control group animals at the 24 and 72 hour sampling times, indicating the correct functioning of the test system. Insufficient numbers of cells were located at the 48 hour sampling time to permit analysis of the data.

1.4 It is concluded that, under the reported experimental conditions, fruttosio-1,6-difosfato administrated intravenously at dose-levels equivalent to 80% and 40% of the LD50 value (700 and 350 mg/kg bodyweight) does not induce micronuclei in the polychromatic erythrocytes of treated mice.

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INTRODUCTION

2.1 Purpose

The work described in this report was conducted to investigate the clastogenic potential of fruttosio-1,6-difosfato, by assessment of its ability to induce micronuclei in the bone marrow erythrocytes of treated mice.

The experiment was performed to comply with the principles of Good Laboratory Practice for non-clinical laboratory studies as set forth by the U.S. Food and Drug Administration. In addition the study was designed to comply with the experimental methods indicated in:

- EEC Council Directive 79/831, Annex V, Part B.
- OECD Guideline for the testing of chemicals No. 474.
- TSCA Test Guidelines issued by the US EPA in 40 CFR part 798 on the 27-Sep-1985 and revised 14-Jan-1986 (Section 798.5395 In vitro mammalian bone marrow cytogenetics tests: Micronucleus test).

2.2 Study organisation

Location of Study

Genetic Toxicology Department
Life Science Research Roma Toxicology Centre
Via Tito Speri, 14
00040 Pomezia (Roma) Italy

Principal dates

Study commenced: 3-Nov-1988
Study completed: 25-Jan-1989

Study Director

P. Mosesso Bs.D.

Archiving

The original data arising from this study, a sample of the test material, microscope slides prepared, and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a period of at least five years from the date of consignment of the report.

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MATERIALS AND METHODS

3.1 Test substance

Five vials of the test material fruttosio-1,6-difosfato (synonym = Esafosfina batch 393/B/APR/88) each containing 5 grams were received from Biomedica Foscama/IRFI on 9-Jun-1988. The test material, which was a fine white powder, was contained in clear glass septum-cap vials labelled with the identity, composition, net weight, batch number and instructions for administration. The test material was stored at 4°C in the dark. Information received from the Sponsor indicated the expiry date as April 1993. All dose-levels in this report are expressed to three significant figures.

3.2 Control substances

The vehicle used in this study was injectable grade distilled water obtained from Laboratori Don Baxter S.p.A., Trieste.

Solutions of Mitomycin-C (Batch 512/AHD: Kiowa Hakko Kogyo Co. Ltd., Tokyo) in distilled water and Busulfan (Batch 211333 584: Fluka AG, Buchs Switzerland) in corn oil were prepared immediately prior to dosing, and served as positive controls.

3.3 Animals

Male and female Swiss CD-1 mice were received in good health from Charles River Italia S.p.A., Calco, Italy on 3 Nov 1988. On the following day the animals had a bodyweight range of 22-29 grams. The animals were allocated to groups immediately on arrival, earmarked on the day of allocation and individually identified by tail marks on the day prior to treatment. Animals were acclimatised and quarantined for twelve days before treatment on 15 Nov 1988.

The day prior to treatment, the male and female animals had bodyweight ranges of 28-41 grams and 25-32 grams respectively.

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3.4 Methods

The methods used were in compliance with the attached Study Protocol.

Previous observations indicated that the positive control Mitomycin-C has a severe toxic effect at the 72 hour sampling time and that consequently increases in the frequency of micronucleated PCE's cannot be observed at this sampling time. In an attempt to obtain an appropriate positive control, treatment with Busulphan at 80 mg/kg was substituted for Mitomycin-C for the 72 hour sampling time.

The bone marrow cell suspension from one animal in the vehicle control group (Animal No.: 47) was lost during laboratory manipulations, and no data is available for this animal.

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MICRONUCLEUS TEST

4.1 Selection of dose-levels

The dose-levels administered to the animals are usually selected as eighty percent and forty percent of the LD50 value respectively. The information received from the Sponsor gave the intravenous LD50 value as 874.5 mg/kg. On the basis of this information the dose-levels were calculated as 700 and 350 mg/kg for both males and females. The intravenous route of administration was selected for this study, on the instructions of the Sponsor. The positive control Mitomycin-C and Busulfan were administered intravenously and intraperitoneally respectively.

4.2.1 Treatment

Preparations of the test compound, positive control substances or vehicle were administered once to groups of 5 male and 5 female mice. At each treatment-level, groups were sacrificed at 24, 48 and 72 hours after treatment. Details of the treatment schedule are given in the following table:

Group	Colour code	Treatment	Dosage mg/kg	Animal numbers Males	Animal numbers Females	Sampling time
1	White	Vehicle	0.00	2-10	1-9	24 hrs
				42-50	41-49	48 hrs
				82-90	81-89	72 hrs
2	Yellow	Test Substance	350	12-20	11-19	24 hrs
				52-60	51-59	48 hrs
				92-100	91-99	72 hrs
3	Blue	Test Substance	700	22-30	21-29	24 hrs
				62-70	51-59	48 hrs
				102-110	101-109	72 hrs
4	Red	Mitomycin-C	5.00	32-40	31-39	24 hrs
				72-80	71-79	48 hrs
4	Red/Black	Busulfan	80.0	112-120	111-119	72 hrs

Reserve animals were treated at the high dose-level to allow substitution in the case of mortalities.

4.2.2 Observations

Animals were inspected daily throughout the study for signs of reaction to treatment. Only minor signs of toxicity were observed; these included urogenital soiling and ungroomed appearance. Three animals died following treatment at the high dose-level and were substituted by reserve animals as detailed:

Animal 21 substituted by reserve animal 121
Animal 64 substituted by reserve animal 122
Animal 70 substituted by reserve animal 124.

4.3 Sacrifice and slide preparation

Groups of 5 male and 5 female animals were sacrificed 24, 48 and 72 hours after the commencement of treatment. The femurs were removed and bone marrow cells obtained by flushing with foetal calf serum. The cells were centrifuged and a concentrated suspension prepared to make smears on slides. These slides were air-dried overnight and then stained with May-Gruenwald and Giemsa, and mounted with Eukitt. Three slides were made from each animal.

4.4 Slide evaluation

The slides were randomly coded by a person not involved in the subsequent microscope scoring. The slides were examined under medium magnification and one slide from each animal was selected according to staining and quality of smears. Where the toxicity of the test substance was not so great as to inhibit cell proliferation, at least 1000 PCE's were examined at high magnification (100x) for the presence or absence of micronuclei. At the same time the number of normal and micronucleated normochromatic erythrocytes was also recorded.

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RESULTS

5.1 Incidence of micronucleated cells

The individual scores for normal and micronucleated, mature and polychromatic erythrocytes for each animal at 24, 48 and 72 hour sampling times are presented in Tables 1, 2 and 3 respectively. In Tables 4, 5 and 6 the calculated incidence of micronucleated cells per 1000 erythrocytes (mature or polychromatic) are given as group means by sex, and for the sexes combined. The standard error of the means and ranges are also shown. The animals presenting less than 200 PCE's per 1000 NE's scored were excluded from the calculations and subsequent statistical analyses.

No marked increases in the numbers of micronucleated PCE's were observed in any fruttosio-1,6-difosfato treatment group at any dose-level within any sampling time.

Pronounced increases in the frequency of micronucleated PCE's were observed in the positive control groups using Mitomycin-C at the 24 hour sampling time indicating the correct functioning of the test system. Insufficient cells were located at the 48 hour sampling time to permit analysis of the data. Treatment with Busulfan resulted in small increases in the frequency of micronucleated PCE's which were more pronounced in female animals.

5.2 Ratio of mature to polychromatic erythrocytes

Slight increases in the ratio of mature to polychromatic erythrocytes (NCE's to PCE's) were observed at the 24 hour sampling time, following treatment with fruttosio-1,6-difosfato. This suggested that the test material was slightly inhibitory to bone marrow erythropoietic cell division.

Marked increases in the ratio of NCE's to PCE's were observed in the positive control groups at the 24 hour sampling time. At the 48 hour sampling time, increases were so severe that insufficient cells were located to calculate a reliable value, while at the 72 hour sampling time, only slight increases were observed.

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ANALYSIS OF RESULTS

6.1 Introduction

The test substance is considered to induce micronuclei if a statistically significant increase in the micronucleus incidence in polychromatic erythrocytes (at $P<0.05$) is observed in any treatment group, in the pooled data for both sexes, or for either sex considered separately. The statistical methods employed are described in Appendix II.

Only counts obtained from polychromatic cells were subjected to statistical analysis. Using the original observations(and not the micronucleus frequencies per 1000 cells), a modified Chi-squared calculation was employed to compare treated and control groups. The degree of heterogenity within each group was first calculated and where this was significant it was taken into account in the comparison between groups. Variance ratios or Chi-squared values are taken to show the significance of any difference between each treated group and the controls. Animals with less than 200 polychromatic erythrocytes out of 1000 normochromatic erythrocytes scored were excluded from the statistical analysis.

Tables 7, 8 and 9 show analyses of the results, examining the results from male and female animals combined and separately, at the 24, 48 and 72 hour sampling times respectively.

6.2 Statistical analysis: combined male and female data

No statistically significant increases in the incidence of micronucleated PCE's (compared with the vehicle control group) were observed in any fruttosio-1,6-difosfato treatment group at any sampling time.

Statistically significant increases were observed in the positive control groups at the 24 and 72 hour sampling times.

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6.3 Statistical analysis by sex

Tables 7, 8 and 9 also show analysis by sex for each sampling time. The results for male and female treatment groups considered separately were compared at each sampling time with the relevant vehicle controls.

No statistically significant increases in the incidence of micronucleated PCE's (compared with the vehicle control group) were observed in any fruttosio-1,6-difosfato treatment group for male or female animals considered separately.

Statistically significant increases were observed in female animals only following treatment with Busulfan at the 72 hour sampling time.

No statistically significant sex-related differences in response were observed at any test substance treatment-level.

6.4 Conclusions

It is concluded that, under the reported experimental conditions, fruttosio-1,6-difosfato administered intravenously at dose-levels of 350 and 700 mg/kg bodyweight does not induce micronuclei in the polychromatic erythrocytes of treated mice.

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CONCLUSIONS

A summary of the results obtained at each sampling time is presented in Table 10, which shows the mean incidence of micronucleated PCE's for each group (combining the data for both sexes), the standard error of the mean and the range of values observed. Statistically significant increases in micronucleated PCE incidence are indicated and the mean NCE/PCE ratio for each group is also shown.

It is concluded that, under the reported experimental conditions, fruttosio-1,6-difosfato administered intravenously at dose-levels equivalent to 80% and 40% of the LD50 value (700 and 350 mg/kg bodyweight) does not induce micronuclei in the polychromatic erythrocytes of treated mice.

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KEY TO TABLES 1-3

These tables present the data obtained for each individual animal in the study.

The results are presented in turn for the 24, 48 and 72 hour sampling times.

In each table, the results are presented for the

Vehicle control group
Low dose-level group
High dose-level group
Positive control group

reading down the page.

The results for male animals are presented on the left hand side of the page, and females on the right hand side.

Abbreviations

Mn : Cells with micronuclei

Tot. PCE: Total number of Polychromatic erythrocytes analysed

Tot. NCE: Total number of Normochromatic erythrocytes analysed

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SCHEDULE NO.: 003-004 Table 1

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - INDIVIDUAL OBSERVATIONS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 24 hours

MALES					FEMALES				
Dose-level mg/kg:		males	0.00		/ females	0.00			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
2	0	1007	0	841	1	1	1097	2	1208
4	1	1077	1	1398	3	1	1059	0	1019
6	0	1033	0	1150	5	1	1011	0	1008
8	1	1016	1	1137	7	0	1051	0	793
10	2	1079	1	991	9	2	1103	0	840

MALES					FEMALES				
Dose-level mg/kg:		males	350		/ females	350			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
12	2	1034	0	1135	11	0	1082	1	1201
14	0	1008	1	1131	13	0	1061	0	867
16	0	1010	0	918	15	1	1063	1	1160
18	1	1077	0	853	17	0	1000	0	804
20	0	1077	1	1191	19	1	1005	0	1158

MALES					FEMALES				
Dose-level mg/kg:		males	700		/ females	700			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
22	0	1002	1	1283	21	0	1006	1	1287
24	2	1026	0	1621	23	1	1052	0	903
26	1	1045	0	1032	25	0	1028	2	1259
28	1	1014	0	1143	27	0	1044	1	1200
30	0	1026	0	1268	29	2	1002	0	1360

MALES					FEMALES				
Mitomycin C 5 mg/kg									
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
32	33	1030	0	1358	31	25	1003	1	1828
34	51	1012	2	1351	33	51	1028	1	1324
36	46	1007	1	1274	35	43	1011	2	1224
38	47	1037	2	1538	37	3	1018	0	1130
40	29	1012	0	1214	39	52	1011	2	1459

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SCHEDULE NO.: 003-004 Table 2

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - INDIVIDUAL OBSERVATIONS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 48 hours

M A L E S					F E M A L E S				
Dose-level mg/kg: males 0.00					/ females 0.00				
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
42	0	1003	0	798	41	1	1033	0	983
44	2	1016	1	886	43	2	1129	0	917
46	1	1054	0	1117	45	1	1019	0	702
48	1	1015	1	994	47		NO DATA		
50	1	1075	0	1072	49	0	1027	0	884

M A L E S					F E M A L E S				
Dose-level mg/kg: males 350					/ females 350				
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
52	1	1009	0	771	51	1	1018	2	1029
54	1	1017	0	758	53	1	1034	1	832
56	0	1093	0	783	55	3	1045	1	933
58	0	1012	1	1078	57	1	1059	1	1120
60	1	1067	2	1143	59	1	1002	1	767

M A L E S					F E M A L E S				
Dose-level mg/kg: males 700					/ females 700				
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
62	0	1048	2	956	61	2	1076	1	1030
64	2	1008	0	1038	63	2	1037	1	702
66	2	1082	3	1067	65	2	1095	0	708
68	1	1012	0	858	67	3	1026	1	958
70	1	1067	0	751	69	0	1014	0	1088

M A L E S					F E M A L E S				
Mitomycin C 5 mg/kg									
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
72	14	55	12	1047	71	16	84	7	1064
74	14	89	17	1018	73	10	67	8	1057
76	6	18	17	1025	75	9	38	12	1024
78	10	74	9	1039	77	17	79	13	1003
80	15	60	17	1024	79	15	195	12	1086

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SCHEDULE NO.: 003-004 TABLE 3

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - INDIVIDUAL OBSERVATIONS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 72 hours

MALES					FEMALES				
Dose-level mg/kg:		males	0.00		/ females	0.00			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
82	2	1028	0	922	81	1	1090	0	859
84	1	1014	1	1336	83	0	1029	1	803
86	0	1040	0	848	85	1	1067	0	874
88	1	1022	1	1183	87	1	1017	1	842
90	0	1040	1	1014	89	1	1081	0	716

MALES					FEMALES				
Dose-level mg/kg:		males	350		/ females	350			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
92	3	1072	0	1077	91	1	1066	1	929
94	1	1041	1	970	93	1	1029	0	767
96	1	1021	0	1093	95	0	1068	0	868
98	3	1002	0	1112	97	2	1098	1	830
100	1	1012	1	1098	99	1	1017	0	823

MALES					FEMALES				
Dose-level mg/kg:		males	700		/ females	700			
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
102	1	1091	1	794	101	1	1074	0	833
104	1	1038	1	764	103	1	1021	1	910
106	1	1071	0	845	105	2	1053	0	881
108	2	1068	0	1074	107	2	1055	2	982
110	1	1058	0	1272	109	1	1032	0	776

MALES					FEMALES				
Busulfan 80 mg/kg									
Animal no.	Mn	Tot.PCE	Mn	Tot.NCE	Animal no.	Mn	Tot.PCE	Mn	Tot.NCE
112	0	1054	0	802	111	5	1024	3	1833
114	0	0	11	1010	113	0	0	8	1036
116	4	1049	2	1322	115	0	0	5	1011
118	1	89	8	1021	117	6	15	10	1158
120	0	1015	1	1181	119	7	1009	3	1449

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KEY TO TABLES 4-6

These tables present the values obtained for each treatment group in the study. The results are presented in turn for the 24, 48 and 72 hour sampling times. In each table, the values are given for males only, females only, and finally the combined data for both sexes.

The values presented are:

Dose-level (mg/kg)	When two values are given, the first value refers to male animals and the second to females.
Scored Cells - PCE	The total number of PCE's scored.
Scored Cells - NCE	The total number of NCE's scored.
NCE/PCE ratio	The ratio of NCE's/PCE's calculated as the mean of the ratio values for the individual animals.

POLYCHROMATIC/NORMOCHROMATIC

- MEAN	The group mean incidence of micronucleated PCE's/NCE's.
- SE	The standard error of the mean incidence.
- MIN	Minimum value observed in an individual animal.
- MAX	Maximum value observed in an individual animal.

SCHEDULE NO.: 003-004 Table 4

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - SUMMARY OF INCIDENCE OF MICRONUCLEATED CELLS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 24 hours

M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	5212	5517	1.06	0.8	0.3	0.0	1.9	0.5	0.2	0.0	1.0
350	5206	5228	1.01	0.6	0.4	0.0	1.9	0.3	0.2	0.0	0.9
700	5113	6347	1.24	0.8	0.4	0.0	1.9	0.2	0.2	0.0	0.8
Mitomycin C 5.00	5098	6735	1.32	40.4	4.2	28.7	50.4	0.7	0.3	0.0	1.5

F E M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	5321	4868	0.92	0.9	0.3	0.0	1.8	0.3	0.3	0.0	1.7
350	5211	5190	0.99	0.4	0.2	0.0	1.0	0.3	0.2	0.0	0.9
700	5132	6009	1.17	0.6	0.4	0.0	2.0	0.6	0.3	0.0	1.6
Mitomycin C 5.00	5071	6965	1.37	34.3	9.1	2.9	51.4	0.9	0.3	0.0	1.6

B O T H S E X E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	10533	10385	0.99	0.8	0.2	0.0	1.9	0.4	0.2	0.0	1.7
350 / 350	10417	10418	1.00	0.5	0.2	0.0	1.9	0.3	0.1	0.0	0.9
700 / 700	10245	12356	1.21	0.7	0.3	0.0	2.0	0.4	0.2	0.0	1.6
Mitomycin C 5.00	10169	13700	1.35	37.4	4.9	2.9	51.4	0.8	0.2	0.0	1.6

SCHEDULEW NO.: 003-004 Table 5

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - SUMMARY OF INCIDENCE OF MICRONUCLEATED CELLS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 48 hours

M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	5163	4867	0.94	1.0	0.3	0.0	2.0	0.4	0.3	0.0	1.1
350	5198	4533	0.87	0.6	0.2	0.0	1.0	0.5	0.4	0.0	1.7
700	5217	4670	0.90	1.2	0.4	0.0	2.0	1.0	0.6	0.0	2.8
Mitomycin C 5.00	INSUFFICIENT CELLS LOCATED										

F E M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	4208	3486	0.83	0.9	0.4	0.0	1.8	0.0	0.0	0.0	0.0
350	5158	4681	0.91	1.4	0.4	0.9	2.9	1.3	0.2	0.9	1.9
700	5248	4486	0.86	1.7	0.5	0.0	2.9	0.7	0.3	0.0	1.4
Mitomycin C 5.00	INSUFFICIENT CELLS LOCATED										

B O T H S E X E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	9371	8353	0.89	1.0	0.2	0.0	2.0	0.2	0.2	0.0	1.1
350 / 350	10356	9214	0.89	1.0	0.2	0.0	2.9	0.9	0.2	0.0	1.9
700 / 700	10465	9156	0.88	1.4	0.3	0.0	2.9	0.8	0.3	0.0	2.8
Mitomycin C 5.00	INSUFFICIENT CELLS LOCATED										

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SCHEDULE NO.: 003-004 Table 6

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - SUMMARY OF INCIDENCE OF MICRONUCLEATED CELLS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 72 hours

M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	5144	5303	1.03	0.8	0.4	0.0	1.9	0.5	0.2	0.0	1.0
350	5148	5350	1.04	1.7	0.5	1.0	3.0	0.4	0.2	0.0	1.0
700	5326	4749	0.89	1.1	0.2	0.9	1.9	0.5	0.3	0.0	1.3
Busulfan 80.00	3118	3305	1.06	1.3	1.3	0.0	3.8	0.8	0.4	0.0	1.5

F E M A L E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	5284	4094	0.78	0.8	0.2	0.0	1.0	0.5	0.3	0.0	1.2
350	5278	4217	0.80	0.9	0.3	0.0	1.8	0.5	0.3	0.0	1.2
700	5235	4382	0.84	1.3	0.2	0.9	1.9	0.6	0.4	0.0	2.0
Busulfan 80.0	2033	3282	1.61	5.9	1.0	4.9	6.9	1.9	0.2	1.6	2.1

B O T H S E X E S

DOSE-LEVEL mg/kg	SCORED CELLS PCE	NCE	NCE/PCE RATIO	INCIDENCE OF MICRONUCLEI PER 1000 CELLS POLYCHROMATIC MEAN	SE	MIN	MAX	NORMOCHROMATIC MEAN	SE	MIN	MAX
0.00	10428	9397	0.90	0.8	0.2	0.0	1.9	0.5	0.2	0.0	1.2
350 / 350	10426	9567	0.92	1.3	0.3	0.0	3.0	0.4	0.2	0.0	1.2
700 / 700	10561	9131	0.86	1.2	0.1	0.9	1.9	0.6	0.2	0.0	2.0
Busulfan 80.0	5151	6587	1.28	3.1	1.4	0.0	6.9	1.2	0.4	0.0	2.1

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KEY TO TABLES 7-9

These tables present the statistical analyses for the 24, 48 and 72 hour groups in turn. The methods are described in detail in section 6 of the report, and in Appendix II.

Each table is composed of 4 sections:

(i) Analysis of the combined data for both sexes.

(ii) Analysis of the data obtained from male animals alone.

(iii) Analysis of the data obtained from female animals alone.

(iv) Analysis for statistically significant differences between the responses of the two sexes.

The chi-squared statistic (X2) and significance level (Sign) are presented for within-group heterogeneity.

The chi-squared (X2) of F-statistic (F), and significance level (Sign) are shown for the comparison between the control and treatment group (or between males and females in the same treatment groups, as appropriate).

NC	Not calculated
NS	Not significant
*	Statistically significant at P<0.05
**	Statistically significant at P<0.01
***	Statistically significant at P<0.001

SCHEDULE NO.: 003-004 Table 7

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - STATISTICAL ANALYSIS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 24 hours

STATISTICAL ANALYSIS - BOTH SEXES

DOSE-LEVEL mg/kg		WITHIN ANIMALS OF ONE GROUP		BETWEEN EACH GROUP AND CONTROL GROUP			
Males	Females	X2	Sign.	X2	Sign.	F	Sign.
0.00	0.00	5.16	N.S.				
350	350	9.03	N.S.	1.10	N.S.		
700	700	8.79	N.S.	0.20	N.S.		
Mitomycin C	5 mg/kg	59.92	***			103.49	***

MALES ONLY

0.00	0.00	3.33	N.S.				
350	350	5.33	N.S.	0.14	N.S.		
700	700	3.47	N.S.	0.00	N.S.		
Mitomycin C	5 mg/kg	9.44	N.S.	202.96	***		

FEMALES ONLY

0.00	0.00	1.89	N.S.				
350	350	3.04	N.S.	1.22	N.S.		
700	700	5.46	N.S.	0.43	N.S.		
Mitomycin C	5 mg/kg	51.16	***			25.76	**

DIFFERENCES BETWEEN SEXES

		BETWEEN MALE AND FEMALE GROUPS		
0.00	0.00	0.09	N.S.	
350	350	0.20	N.S.	
700	700	0.15	N.S.	
Mitomycin C	5 mg/kg			0.35 N.S.

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SCHEDULE NO.: 003-004 Table 8

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - STATISTICAL ANALYSIS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 48 hours

STATISTICAL ANALYSIS - BOTH SEXES

DOSE-LEVEL mg/kg		WITHIN ANIMALS OF ONE GROUP		BETWEEN EACH GROUP AND CONTROL GROUP			
Males	Females	X2	Sign.	X2	Sign.	F	Sign.
0.00	0.00	3.80	N.S.				
350	350	5.97	N.S.	0.00	N.S.		
700	700	5.65	N.S.	0.92	N.S.		
Mitomycin C	5 mg/kg		N.C.		N.C.		N.C.

MALES ONLY

0.00	0.00	2.02	N.S.				
350	350	2.05	N.S.	0.51	N.S.		
700	700	2.34	N.S.	0.08	N.S.		
Mitomycin C	5 mg/kg		N.C.		N.C.		N.C.

FEMALES ONLY -

0.00	0.00	1.78	N.S.				
350	350	2.22	N.S.	0.33	N.S.		
700	700	2.67	N.S.	0.99	N.S.		
Mitomycin C	5 mg/kg		N.C.		N.C.		N.C.

DIFFERENCES BETWEEN SEXES

			BETWEEN MALE AND FEMALE GROUPS		
0.00	0.00		0.00	N.S.	
350	350		1.63	N.S.	
700	700		0.58	N.S.	
Mitomycin C	5 mg/kg	N.C.		N.C.	N.C.

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SCHEDULE NO.: 003-004 Table 9

LSR-RTC DEPARTMENT OF GENETIC TOXICOLOGY
MICRONUCLEUS TEST - STATISTICAL ANALYSIS

TEST SUBSTANCE : Fruttosio-1,6-difosfato
SAMPLING TIME : 72 hours

STATISTICAL ANALYSIS - BOTH SEXES

DOSE-LEVEL mg/kg		WITHIN ANIMALS OF ONE GROUP	BETWEEN EACH GROUP AND CONTROL GROUP	
Males	Females	X2 Sign.	X2 Sign.	F Sign.
0.00	0.00	4.54 N.S.		
350	350	6.00 N.S.	1.64 N.S.	
700	700	1.60 N.S.	1.13 N.S.	
Busulfan	80.0 mg/kg	12.44 *		9.39 **

MALES ONLY

0.00	0.00	3.53 N.S.		
350	350	2.62 N.S.	1.92 N.S.	
700	700	0.66 N.S.	0.33 N.S.	
Busulfan	80.0 mg/kg	7.90 *		0.27 N.S.

FEMALES ONLY

0.00	0.00	0.97 N.S.		
350	350	1.90 N.S.	0.11 N.S.	
700	700	0.83 N.S.	0.85 N.S.	
Busulfan	80.0 mg/kg	0.37 N.S.	17.82 ***	

DIFFERENCES BETWEEN SEXES

		BETWEEN MALE AND FEMALE GROUPS	
0.00	0.00	0.00 N.S.	
350	350	1.25 N.S.	
700	700	0.10 N.S.	
Busulfan	80.0 mg/kg		3.08 N.S.

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SCHEDULE NO.: 003-004

Table 10

MICRONUCLEUS TEST
SUMMARY TABLE

TEST SUBSTANCE : Fruttosio-1,6-difosfato
ROUTE OF ADMINISTRATION: Intravenous
VEHICLE : Sterile distilled water

Treatment	Dose-level (mg/ml)	Incidence of Micronucleated PCE's Mean ± SE	Range	NCE/PCE Mean Ratio
24 hr sampling time				
Vehicle	10 ml/kg	0.8 ± 0.2	0.0 - 1.9	0.99
Test Substance	350	0.5 ± 0.2	0.0 - 1.9	1.00
Test Substance	700	0.7 ± 0.3	0.0 - 2.0	1.21
Mitomycin-C	5.00	37.4 ± 4.9***	2.9 - 51.4	1.35
48 hr sampling time				
Vehicle	10 ml/kg	1.0 ± 0.2	0.0 - 2.0	0.89
Test Substance	350	1.0 ± 0.2	0.0 - 2.9	0.89
Test Substance	700	1.4 ± 0.3	0.0 - 2.9	0.88
Mitomycin-C	5.00	Insufficient cells located		
72 hr sampling time				
Vehicle	10 ml/kg	0.8 ± 0.2	0.0 - 1.9	0.90
Test Substance	350	1.3 ± 0.3	0.0 - 3.0	0.92
Test Substance	700	1.2 ± 0.1	0.9 - 1.9	0.86
Busulfan	80.0	3.1 ± 1.4**	0.0 - 6.9	1.28

Key:
PCE : Polychromatic erythrocyte
NCE : Normochromatic erythrocyte
* : Incidence significantly greater than control value at $p<0.05$
** : Incidence significantly greater than control value at $p<0.01$
*** : Incidence significantly greater than control value at $p<0.001$

APPENDIX I

STUDY PROTOCOL

LIFE SCIENCE RESEARCH
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(Dr. ALFREDO NUNZIATA)

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.P.A.

LSR-RTC Enquiry no. 1471

MICRONUCLEUS TEST
Test Substance: ESAFOSFINA

Protocol prepared for

BIOMEDICA FOSCAMA
IRFI.
Via Morolese, 87
03013 Ferentino (FR)

by

Life Science Research
Roma Toxicology Centre
Via Tito Speri 14
Pomezia (Roma)

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.P.A.

MICRONUCLEUS TEST
PROTOCOL APPROVAL

For LIFE SCIENCE RESEARCH - ROMA TOXICOLOGY CENTRE

Issued by : . . . . . date: . 19. May. 88

Released by: . . . . . date: . . 19. May. 88.

For BIOMEDICA FOSCAMA

This protocol is accepted without revision and my signature authorises the study to proceed as described in this document. The document becomes the FINAL PROTOCOL for the study, and will be reproduced in the final report.

Approved by: . . . . . date: 9. 6. 88. . .

STUDY DIRECTOR

The Sponsor has approved the initiation of this study according to the procedures described in this document. My signature below denotes that I have read and agreed the contents of this document.

Agreed by : . . . . . date: .14 JUNE 1988. .
(P. Mosesso Bs.D., Study Director)

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(Dr. ALFREDO NUNZIATA)

MICRONUCLEUS TEST

MANAGEMENT OF STUDY

Head Department of Genetic Toxicology	: R. Forster, M.A. (Cantab.), Ph.D.
Person Responsible to Ministry of Health	: A. Nunziata, Pharm.D., Chem.D.
Study Director	: P. Mosesso Bs.D.
Sponsor	: BIOMEDICA FOSCAMA IRFI. Via Morolese,87 03013 Ferentino (FR)
Monitor	: To be designated by the Sponsor.

QUALITY ASSURANCE

Quality Assurance Manager : V. Sforza B.Sc.

LOCATION OF STUDY

The study will be performed at:

Life Science Research Roma Toxicology Centre
Via Tito Speri, 14
00040 Pomezia, ROMA

The laboratory facilities, archives and administration are located at this site.

TIME SCHEDULE OF STUDY

The study will be conducted with a time schedule agreed between the Sponsor and LSR-RTC.

TEST SUBSTANCE IDENTITY

The test substance will be : **ESAFOSFINA**

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MICRONUCLEUS TEST

1. INTRODUCTION

1.1 Objective

To assay the test substance for the ability to induce chromosomal damage in mouse bone marrow, as measured by the induction of micronuclei in polychromatic erythrocytes.

1.2 Regulatory requirements

This study is designed as a screening assay (as described in Annex V of EEC Council Directive 79/831). Instead of the single dose-level required by the EEC guideline protocols, two dose-levels are used; in this way useful data may be obtained from the study even if excessive lethality or toxicity is observed at the high dose-level.

If a confirmatory assay is required (as defined by the EEC Directive) then an alternative protocol should be requested, in which three dose-levels are employed.

The study is similarly designed to comply with the scientific requirements of:

- OECD Guideline for the testing of chemicals No. 474
- TSCA Test Guidelines issued by the US EPA in 40 CFR part 798 on 27-Sept-1985 and revised on 14-Jan-1986 (Section 798.5395 In vivo mammalian bone marrow cytogenetics tests: Micronucleus test).

The study will also be performed in compliance with the principles of Good Laboratory Practice, as set forth by the US Food and Drug Administration.

1.3 Principles of the method

The micronucleus test provides a relatively rapid method for investigating the ability of chemicals to induce chromosomal damage or damage to the mitotic apparatus. Because it offers a convenient method of screening for clastogenic properties, the test has been widely used, and an extensive data base is available for the evaluation of the assay's performance in detecting mutagens and carcinogens. Although the test can be performed using a range of animal species and tissues, the test system of choice has been the newly formed erythrocyte in mouse bone marrow.

In this protocol the test substance is administered in vivo to mice once, and bone marrow samples are taken at 24h, 48h and 72h. Microscope slides are prepared from the femoral bone marrow. The slides are scored for the presence of micronuclei in polychromatic erythrocytes.

Micronuclei are small secondary nuclei which originate in mitotically dividing cells, from fragments of damaged chromosomes, or as the result of non-disjunction events. When erythroblasts develop into erythrocytes, the main nucleus is expelled while the micronucleus may remain in the cytoplasm, where it can readily be identified. Micronuclei occur rarely in normal dividing cells, but greater numbers are induced in cells taken from animals exposed to known clastogens.

The mouse is a suitable laboratory animal for the performance of this test, and has a well established genetic background.

2. TEST AND CONTROL SUBSTANCES

2.1 It is the responsibility of the Sponsor to supply the test substance, accompanied by analytical data confirming the identity, purity, stability, strength and composition of the substance, the solubility and stability in the proposed vehicle and details of any known hazards to laboratory staff.

2.2 To comply with the requirements of the Italian Ministry of Health, the test substance should be accompanied by a certificate of analysis, and a sample will be retained in the archives for a period of five years after the completion of the study.

2.3 The test substance identity is indicated on previous pages of this protocol.

2.4 Unless otherwise indicated by the Sponsor, the storage conditions for the test substance will be 4°C in the dark.

2.5 The test substance will be allocated a hazard rating according to the LSR-RTC Hazard Classification system, and handled using appropriate safety precautions.

2.6 The amount of test substance received and used will be recorded according to standard procedures.

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2.7 Fresh solutions of the test substance will be prepared for each day's work; solutions will be prepared on a weight/volume basis without correction for the displacement due to the volume occupied by the test substance. Unless specified by the Sponsor, concentrations of solutions will be expressed in terms of material as received, and not of active constituents. Preferred vehicles will be: physiological saline, buffers, sterile water, 0.5% carboxymethylcellulose (CMC), olive oil or corn oil. Other vehicles may be used as necessary.

2.8 No assay of test substance stability, nor its concentration and homogeneity in vehicle will be undertaken, nor samples of formulated test substance consigned to the Sponsor, without express instructions from the Sponsor. No determination of the absorption of the test substance in the test system will be made without express instructions from the Sponsor.

2.9 Positive control treatments use the well-known clastogen Mitomycin-C. This is obtained commercially and characterised by its labelling. Solutions are prepared freshly for use: the vehicle used will be either the vehicle used for test substance, or isotonic saline or 0.5% CMC, as indicated by the Study Director. Determination of the stability and concentration of solutions of this agent will not be undertaken without express instructions from the Sponsor.

3. EXPERIMENTAL DESIGN

In this study, groups of animals (5 male, 5 female) are treated once with either the vehicle alone (vehicle controls), the test material or the clastogen Mitomycin-C (positive control group). Two treatment levels of the test material are used. At three subsequent sampling times the animals are sacrificed, and slides prepared from the femoral bone-marrow for the scoring of micronuclei.

The experimental design is displayed in tabular form below:

Treatment		Number of Mice					
		24 h		48 h		72 h	
		M	F	M	F	M	
Negative control	Vehicle only	5	5	5	5	5	5
Test substance treatment	0.8 x LD50	5	5	5	5	5	5
Test substance treatment	0.4 x LD50	5	5	5	5	5	5
Positive control	Mitomycin-C (Kiowa) 5 mg/kg	5	5	5	5	5	5

The positive control agent and treatment may be varied at the discretion of the Study Director.

4. TREATMENT AND SELECTION OF DOSE-LEVELS

4.1 Route of administration

The route of administration of the test compound may be by intraperitoneal injection, or by oral gavage using a catheter, according to the proposed clinical use or exposure to the substance. In the absence of specific instructions, the intraperitoneal route will be used.

Negative (vehicle) control animals will receive the selected vehicle only, given by the same route of administration as the test substance. The positive control treatment with Mitomycin-C will be administered via the same route as the test substance.

4.2 Selection of dose-levels

The dose-levels are selected on the basis of the LD50 of the test substance: it is therefore necessary to know the LD50 of the test substance in mice by the appropriate route of administration. The dose-levels used are equivalent to eighty percent and forty percent of the LD50.

If the stated oral LD50 of the test substance exceeds 5 g/kg, the maximum dose-level used in the micronucleus test will be 5 g/kg and the lower dose-level 2.5 g/kg. If the stated intraperitoneal LD50 exceeds 4 g/kg, then the doses for intraperitoneal administration will be selected as 4 and 2 g/kg. The positive control, Mitomycin-C (Kiowa) will be administered at 5 mg/kg, via the selected route of administration.

If detailed acute toxicity data in the mouse is not available the acceptability of the proposed high dose-level may be checked prior to the micronucleus test. The proposed high dose-level will be administered once to a group of two male and two female mice to confirm survival to 72 hours after treatment.

If these animals do not survive to 72 hours or there are other indications that the selected dose-level is inappropriate then the following procedure will be used to select the high dose-level for the study. Further groups of two male and two female animals will be treated once (in the following days) and sacrificed after 24 hours. Bone marrow preparations will be made and examined. The high dose-level will be selected to maximise exposure of the animals to the test material.

5. ASSAY PROCEDURE

5.1 Animal supply

Swiss CD-1 mice of both sexes are obtained from Charles River Italia, Como. Young adult animals (weighing approx. 25-30 grams and aged 5 to 6 weeks at the time of treatment) are used for this study. At this age erythropoietic acitvity is optimal and there is no presence of fat accumulation in the marrow. This factor is not critical, and real differences in sensitivity between animals of different age groups have not been demonstrated.

5.2 Animal husbandry

The animals are housed at 5 animal/cage, by sexes, in clear polycarbonate cages measuring 35.5 x 23.5 x 19 cm with a stainless steel mesh lid and floor (Type 2b: Techniplast). Each cage will hold absorbent bedding which will be inspected daily and changed as necessary. The temperature and relative humidity of the animal rooms are monitored daily. The animals will be kept in a 12 hour light/dark cycle.

Food and drinking water will be supplied ad libitum. The animals are maintained on Altromin MT diet. Quality control aspects of the diet and drinking water are detailed in Addendum 1.

At least five days are allowed for acclimatisation and quarantine; during this period the health status of the animals will be assessed by daily observations. Animals observed to be unfit prior to treatment, will be removed from the study, and may be replaced if stocks allow.

Dated and signed records of activities relating to the day to day running and maintenance of the study in the animal accomodation will be recorded in a Study Day book.

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5.3 Allocation of animals

Shortly after arrival the animals will be uniquely identified by tail or ear markings, and will be randomly allocated to treatment groups. Colour-coded cage labels identifying the occupants by experiment, number, sex and treatment group will be attached to all cages.

5.4 Treatment

Animals to be treated by oral gavage will be starved overnight prior to treatment. After dosing the food hoppers will be refilled.

The appropriate dosage will be calculated for each individual animal, and administered using test solutions or suspensions prepared to deliver an appropriate dosage for each 10 g bodyweight in a volume of 0.1 ml (ie 10 ml/kg). Bodyweight will be determined immediately before treatment. It may be necessary to exceed this volume-dosage for some test materials, in which case the final report will detail the volume-dosage used, and the reasons for exceeding 10 ml/kg.

5.5 Observations

The animals will be inspected regularly throughout the period between treatment and sacrifice for signs of reactions. Animals judged by the Study Director to be in extreme suffering, may be killed for humane reasons. Animals which die during treatment will be removed from the study. They will be subjected to post-mortem examination only as considered necessary.

Group mean body weights will be recorded at the commencement of treatment, and daily throughout the treatment period.

5.6 Extraction of bone marrow

At the appropriate time after treatment, the animals are sacrificed by dislocation of the cervical vertebrae. The femurs of each animal are rapidly dissected out and cleaned of surrounding tissue.

In order to extract the bone marrow, the bone is cut at the proximal end, and irrigated with foetal calf serum using a syringe. The suspension of cells is aspirated, and this procedure is repeated several times.

If the administration of the test material was via the intraperitoneal route, then at sacrifice the peritoneal cavities of some animals will be examined for the presence of precipitated test material.

5.7 Preparation of the smears

The suspension thus obtained is centrifuged at 1000 rpm for 5 min. and the supernatant is completely removed. The cells of the sediment are then resuspended and transferred onto clean microscope slides as smear preparations. They are air-dried overnight and subsequently stained with May-Gruenwald and Giemsa solutions in phosphate buffer (pH 6.8).

5.8 Scoring of the slides and data analysis

For each animal, at least two slides are prepared. These slides are randomised and coded by staff not subsequently involved in the scoring. Provided that the slides are of an adequate quality and a sufficient number of cells can be scored, it is only be necessary to score one of each pair. Scoring is effected using a microscope and high-power objective.

Immature polychromatic erythrocytes (PCE's) stain a basophilic blue-grey colour (since they retain basic ribosomal material for approximately 24 h after enucleation), and can be distinguished from the acidophilic orange-pink normochromatic erythrocytes (NCE's). The polychromatic cells are also slightly larger and have more diffuse boundaries. Erythrocytes lack nuclei, making micronuclei obvious when present; the criteria of Schmid (1976) will be used to score micronuclei.

One thousand polychromatic erythrocytes per animal are scored for the presence of micronuclei (unless there is a marked depression in PCE numbers). At the same time the number of normochromatic erythrocytes is recorded, as well as the number of micronucleated NCE's.

The ratio of PCE's to NCE's gives an indication of the toxicity of the treatment; an increase in the ratio indicates inhibition of cell division. The incidence of micronucleated NCE's gives an indication of the pre-treatment status of the animals. Finally, the incidence of micronucleated PCE's provides an index of induced genetic damage.

5.9 Additional Scoring

Where the Study Director judges it to be necessary, additional scoring of slides which have not been examined, or rescoring of slides which have been examined, may be undertaken after appropriate coding or re-coding of the slides. In such cases the Study Director will document fully the reasons for his decision.

6. REPORTING

6.1 Presentation of Data

The data will be presented in the form of tables. The observations on each individual animal will be displayed (normal and micronucleated PCE's and NCE's) together with the calculated incidence of micronucleated cells per thousand. Treatment group values for the numbers of cells scored, NCE/PCE ratio and incidence of micronucleated PCE's and NCE's will be presented by sexes, and for both sexes combined. The statistical analysis will be tabulated, displaying the calculated statistics for within and between group variation. A summary table will display for each treatment-level and sampling time, the mean incidence of micronucleated PCE's, the level of statistical significance, and the NCE/PCE ratio.

6.2 Statistical analysis of data

Only counts from polychromatic cells are subjected to statistical analysis. Using the original observations (and not the micronucleus frequencies per 1000 cells) a modified chi-squared calculation is employed to compare treated and control groups. The degree of heterogeneity within each group is first calculated, and where it is significant it is taken into account in the comparison between groups.

If there is no significant within-group heterogeneity, the chi-squared test is used to compare treated groups with the controls. If there is significant within-groups heterogeneity, then that group is compared with the controls using a variance ratio (F) value calculated from the between-group and within-group Chi-squared values.

6.3 Evaluation of Results

The test substance will be considered to induce micronuclei if a statistically significant and biologically meaningful increase in micronucleus incidence (at $p<0.05$, after correction for multiple comparisons) is observed in any treatment group, in the pooled data for both sexes, or in the data for male or female groups alone.

The evaluation of data from groups in which there is extensive lethality of the test substance treatment will follow LSR-RTC Standard Operating Procedures. Similarly, where erythropoiesis is depressed by the test substance treatment and few PCE's are available for scoring, evaluation will follow LSR-RTC SOP's. These SOP's follow the recommendations of the US EPA Gene Tox program.

Where increases in the incidence of micronucleated PCE's are observed which are statistically significant, but fall within the range of vehicle control values within this laboratory, then concurrent and historical control data may be used to demonstrate that these increases do not have biological significance.

6.4 Reporting procedure

Unless previously specified by the Sponsor, a Final Report will be issued after the completion of the study. If any corrections or additions are required to the Final Report, these will be in the form of an addendum by the Study Director. The addendum will clearly identify that part of the final report that is being added to or corrected, and the reasons for the changes, and will be signed and dated by the person responsible.

If previously specified by the Sponsor, a Draft Report may be supplied, and a Final Report issued subsequently to include any agreed changes or amendments.

6.5 Final Report

The following information and data will be included in the final report:

- name and address of the facility performing the study and the dates on which the study was initiated and completed;
- objective and procedures stated in the approved protocol, including any approved changes to the original protocol;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- the test article, identified by name, chemical name or chemical number;
- method used;

- any unforeseen circumstances that may have affected the quality or integrity of the study;

- the name and signature of the Study Director;

- a summary of the data, an analysis of the data and a statement of the conclusions drawn for the analysis;

- the location where all raw data, specimens and final report are to be stored.

6.6 Records kept

Full records will be maintained of all aspects of study conduct, along with the results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the study.

6.7 Archiving

All raw data, records and documentation arising from this study, a sample of the test substance, microscope slides, and a copy of the final report consigned will be stored in the archives of Life Science Research - Roma Toxicology Centre for a period of five years from the date of consignment of the report.

7. STUDY CONDUCT

7.1 Language

English language and Italian language version of the study protocol, Standard Operating Procedures and other study documents may be used interchangeably. Similarly, English and Italian renderings of chemical names, including that of the test material will be considered to be equivalent.

7.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test substances. When the study director considers it advisable to modify the procedures described for the selection of a solvent, selection of dose-levels, interpretation of the outcome of the study or other aspects of the study conduct, he will record carefully the decision he has reached and the reasoning which led to it.

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(Dr. ALFREDO NUNZIATA)

7.3 Quality assurance

The study is subjected to the procedure for quality assurance specified in relevant sections of the regulations pertaining to the conduct of Non Clinical Laboratory Studies published by the U.S. Food and Drug Administration. Specifically:

- the protocol is inspected for compliance;
- at least one phase relevant to the study will be inspected;
- procedures and data of the laboratories concerned will be inspected at intervals adequate to assure the integrity of the study;
- the final report is reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in agreement with the raw data;
- periodic reports on these activities are made to management and the Study Director.

All raw data pertaining to this study will be available for inspection by the Study Monitor (for scientific monitoring) or the Quality Assurance Unit of the Sponsor (compliance monitoring).

8. DEPARTURES FROM REGULATORY REQUIREMENTS

Items which are at the responsibility of the Sponsor are indicated in sections 2.1, 2.2, 2.8 and 2.9 of this protocol. Since full compliance with regulatory requirements may depend on the performance of these items, the Sponsor should ensure that appropriate actions are initiated or undertaken.

9. REFERENCES

Heddle, J.A. et al. (1983)
The Induction of Micronuclei as a measure of Genotoxicity. A report of the U.S. Environmental Protection Agency Gene-Tox Program.
Mutation Res. 123, 61-118

Jenssen D. and Ramel C. (1980)
The micronucleus test as a part of a short-term mutagenicity test program for the prediction of carcinogenicity evaluated by 143 agents tested.
Mutation Res. 75, 191-202

Salamone M., Heddle J., Stuart E. and Katz M. (1980)
Towards an improved micronucleus test; studies on 3 model agents, mitomycin-C, cyclophosphamide and dimethylbenzanthracene.
Mutation Res. 74, 347-356.

Schmid W. (1976)
Micronucleus Test for Cytogenetic Analysis.
in: Chemical Mutagens, vol. 4
A. Hollaender (Ed.)
Plenum Press (1976)

Schmid W. (1977)
The Micronucleus Test
in: Handbook of Mutagenicity Test Procedures
B.J. Kilbey et al. (Eds.)
Elsevier North Holland

The collaborative study group for the micronucleus test (1986)
Sex differences in the micronucleus test
Mutation Research 172, 151-163.

Version no.: 87/1

ADDENDUM I

Quality Control aspects of Diet and Drinking Water

1. DIET

The animals are maintained on Altromin MT diet. Altromin MT is a fixed formula rodent diet manufactured by Altromin-Rieper, Bolzano, Italy. The standards of production adopted by the manufacturers have been approved by the LSR-RTC Quality Assurance Manager. The nutritional content is as shown below:

Nutrients	Typical level (%)
Crude protein	23
Crude lipid	5.5
Crude fibre	5.0
Ash	9
Moisture	13

Analyses are made on all batches of diet used to establish the levels of specified substances and micro-organisms likely to be present in feed components and which, if in excess of specified amounts, might have an undesirable effect on the test animals.

Reject levels are based on those quoted in EPA guidelines for the administration of the Toxic Substances Control Act. (USA).

(A) Contaminants	Maximum allowable concentration (ppm)
Total Aflatoxin (B1, B2, G1, G2)	0.005
Lindane	0.02
Heptachlor	0.02
Malathion	2.50
DDT (total)	0.10
Dieldrin	0.02
PCB	0.15
Cadmium	0.48
Arsenic	2.00
Lead	3.00
Mercury	0.20
Selenium	0.60

ADDENDUM I (continued)

(B) Microbial content

Maximum count, at time of manufacture.

Total viable organisms	20,000/g
E.coli	0 in 10g
Salmonella	0 in 50g

In addition LSR-RTC receive estrogenic activity assay results every three months and will monitor levels.

2. DRINKING WATER

Water is taken from the public supply, and conforms to European Council Standards for potable water intended for human consumption (80/778/EEC). At approximately six monthly intervals, samples of water are tested for the chemical quality of the water by screening for the priority pollutants listed below and the microbiological quality of the water is tested.

(A) CHEMICAL CONTAMINANTS

1. Organic materials — Maximum admissible concentration (ppb)

Persistent organochlorine and organophosphorus pesticides.

- substances considered separately	0.1
- total	0.5
- PCB (total)	0.5
- purgeable organochlorine substances including trihalomethanes	1

2. Metals — Maximum admissible concentration (ppm)

Arsenic	0.05	
Cadmium	0.005	
Calcium	100	(guide-level)
Copper	3	(guide-level)
Mercury	0.001	
Lead	0.05	
Selenium	0.01	
Zinc	5	(guide-level)

ADDENDUM 1 (continued)

3. Inorganic ions	Maximum admissible concentration (ppm)
Nitrate	50
Nitrite	0.1

(B) MICROBIOLOGICAL CONTAMINANTS	Maximum admissible content per 100 mls
Total coliforms	0
Faecal coliforms	0
Salmonella	0

The results of the above analyses of the diet and drinking water will be retained in the archives of LSR-RTC, and referenced where appropriate in the study data.

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(Dr. ALFREDO NUNZIATA)

APPENDIX II: STATISTICAL ANALYSIS OF THE DATA

1. Within Group (χ^2_ω)

The variation between individual animals within each treatment group is assessed by calculation of the term χ^2_ω from a table of the form:

Animal No.	No. of micronucleated PCE's.	Total No. of PCE's examined.	Incidence of micronucleated PCE's.
n 1	x 1	m 1	P 1 = x 1/m 1
n 2	x 2	m 2	P 2 = x 2/m 2
n 3	x 3	m 3	P 3 = x 3/m 3
n 4	x 4	m 4	P 4 = x 4/m 4
.	.	.	.
.	.	.	.
n10	x10	m10	P10 = x10/m10
	X	M	$P = \frac{X}{M}$

Where $\chi^2_\omega = \frac{\sum xn \cdot Pn - XP}{P(1-P)}$ with 9 degrees of freedom (dfW)

2. Between each treatment group and the controls

The variation between each treatment group and the control group is evaluated by calculation of the term χ^2_B from a table constructed as follows:

Group No.	No. of micronucleated PCE's.	Total No. of PCE's. examined.	Incidence of micronucleated PCE's.
N1	d1	t1	P1 = d1/t1
N2	d2	t2	P2 = d2/t2
	D	T	P = D/T

Where $\chi^2_B = \frac{\sum dnPn - DP}{P(1-P)}$ with 1 degree of freedom (dfB)

If χ^2_ω is non-significant for both groups (i.e. there is no heterogeneity within either group), only the term χ^2_B is calculated.

APPENDIX III

DIET ANALYSIS

A.Rieper S.p.A.
Via B.V. Guggenberg, 6
39030 Vandoies / BZ

Tel. 0472/49821

LIFE SCIENCE RESEARCH ROMA
TOXICOLOGY CENTRE SPA
VIA TITO SPERI 14
00040 POMEZIA/ROMA

a.m. Dott. Sforza

13.06.88

Oggetto: certificati d'analisi / Lotto nr. 8806
------- bolla n. 6629 del 06.06.88

Allegato alla presente Vi trasmettiamo i certificati d'analisi relativi ai seguenti prodotti:

- ALTROMIN MT 3.750 kg

Distinti saluti

A.Rieper S.p.A.
Molini ed Industria Mangimi

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S
(Dr. ALFREDO NUNZIATA)

Allegati: citati

MT/8806

Landwirtschaftliche Untersuchungs- und Forschungsanstalt Kiel der Landwirtschaftskammer Schleswig-Holstein

Agricultural Analysis and Research Institute, Kiel of the Ministry of Agriculture, Schleswig-Holstein

Landwirtschaftliche Untersuchungs- und Forschungsanstalt Kiel
Postfach 30 67 · 2300 Kiel 1

A. Rieper AG
Mühle
Kraftfutterwerk

I - 39030 Vintl/Bz

Ref: So 13391,So 5298,Ba 5281/87 Schu
(please quote in correspondence)

2300 Kiel 1, 02.06.1988 Schu
Gutenbergstraße 75-77 Tel. (0431) 1 50 87 u. 1 50 88
Telegramm-Adresse: LUFA Kiel
Telex-Nr. 0 292 634 lufak d

The analysis of the sample No. 74 received on 20.05.1988

Definition: Altromin-Rieper Type MT, Batch/Lotto No. 8806

Packing plastic-bag

had the following result:

		Qualitative analysis			Quantitative analysis
Chlorinated hydrocarbons:	Tecnazen	less than	0,001	mg/kg	
	HCB (Hexachlorbenzol)	less than	0,001	mg/kg	
	α - HCH	less than	0,001	mg/kg	
	β - HCH	less than	0,001	mg/kg	
	γ - HCH (Lindan)	~~less than~~	~~0,001~~	~~mg/kg~~	0,002 mg/kg
	δ - HCH	less than	0,001	mg/kg	
	Quintozen	less than	0,001	mg/kg	
	Heptachlor	less than	0,001	mg/kg	
	Heptachlorepoxid	less than	0,003	mg/kg	
	α - Chlordan	less than	0,005	mg/kg	
	γ - Chlordan	less than	0,005	mg/kg	
	α - Endosulfan	less than	0,005	mg/kg	
	β - Endosulfan	less than	0,005	mg/kg	
	Aldrin	less than	0,003	mg/kg	
	Dieldrin	less than	0,003	mg/kg	
	Endrin	less than	0,003	mg/kg	
	o,p-DDE	less than	0,003	mg/kg	
	p,p-DDE	less than	0,003	mg/kg	
	o,p-DDD	less than	0,005	mg/kg	
	o,p-DDT	less than	0,005	mg/kg	
	p,p-DDD	less than	0,005	mg/kg	
	p,p-DDT	less than	0,005	mg/kg	
	Methoxychlor	less than	0,01	mg/kg	

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MT/ 8205

		Qualitative analysis			Quantitative analysis
Phosphoric acid esters:	Chlorthion	less than	0,01	mg/kg	
	Disulfoton	less than	0,005	mg/kg	
	Malathion	~~less than~~	~~0,01~~	~~mg/kg~~	0,082 mg/kg
	Parathion (-methyl)	less than	0,005	mg/kg	
	Parathion (-äthyl)	less than	0,01	mg/kg	
	Sulfotepp	less than	0,002	mg/kg	
	Fenthion	less than	0,005	mg/kg	
	Diazinon	less than	0,01	mg/kg	
	Dimethoate	less than	0,005	mg/kg	
	Trichlorphon	less than	0,01	mg/kg	
	Bromophos (-methyl)	less than	0,01	mg/kg	
	Bromophos (-äthyl)	less than	0,01	mg/kg	
	Chlorfenvinphos	less than	0,01	mg/kg	
	Methidathion	less than	0,01	mg/kg	
	Ethion	less than	0,01	mg/kg	
	Aflatoxin B1:	less than	0,003	mg/kg	
	Aflatoxin B2:	less than	0,003	mg/kg	
	Aflatoxin G1:	less than	0,003	mg/kg	
	Aflatoxin G2:	less than	0,003	mg/kg	
	PCB:	less than	0,01	mg/kg	
	Nitrate:	less than	50	mg/kg	
	Nitrite:	less than	10	mg/kg	
	Arsenic (As):	less than	0,2	mg/kg	
	Lead (Pb):	~~less than~~	~~0,1~~	~~mg/kg~~	0,3 mg/kg
	Cadmium (Cd):	~~less than~~	~~0,01~~	~~mg/kg~~	0,06 mg/kg
	Mercury (Hg):	less than	0,01	mg/kg	
	Selenium (Se):	~~less than~~	~~0,1~~	~~mg/kg~~	0,10 mg/kg
	Fluorine (F):	~~less than~~	~~5,0~~	~~mg/kg~~	17 mg/kg
	Antibiotic activity:	None detected			

62 LANDWIRT-SCHAFTSKAMMER SCHLESWIG-HOLSTEIN

Hoffmann

LIFE SCIENCE RESEARCH CENTRE S.p.A.
ROVA... TOXICOLOGICO
(... FREDO NONZIATA)

20-07-33 MT/33

Dr. med. vet. Richard Hörter
Fachtierarzt für
Mikrobiologie und Serologie
Veterinary specialist in
Microbiology and Serology

D-4930 Detmold
Trakehnerweg 22
Tel. (0 52 31) 8 81 55

27.5.1988

Firma

A.Rieper AG
Kraftfutterwerk

I-39030 Vintl/Bz

Date: 27.5.1988

The sample received for analysis of number and types of germs
on 17.5.1988 2/Lab2/S1 gave the following results:

Sample No.	No. germs per gram	Aerobic germs	Clostridiae	Fungi per gram
87	9 700	not differentiated	pos. (apathogen)	-

Salmonellae /50g n.d.
E.coli /10g n.d.
Streptococcus /10g n.d.
Staphylococcus/10g n.d.

Also, no pests such as mites, bugs nor eggs or larvae thereof, were detectable

Values obtained after 24 hours- incubation

Definition: Altromin-Rieper Type MT Batch/Lotto No. 8806

MT/8803

Dr. med. vet. Richard Hörter
Fachtierarzt für
Mikrobiologie und Serologie
Veterinary specialist in
Microbiology and Serology

D-4930 Detmold
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27.5.1988

Firma
A.Rieper AG
Kraftfutterwerk

I-39030 Vintl/Bz

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87	9 700	not differentiated	pos. (apathogen)	-

Salmonellae /50g n.d.
E.coli /10g n.d.
Streptococcus /10g n.d.
Staphylococcus/10g n.d.

Also, no pests such as mites, bugs nor eggs or larvae thereof, were detectable

Values obtained after 24 hours- incubation

Definition: Altromin-Rieper Type MT, Batch/Lotto No. 880F

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALBERTO NUNZIATA)

MT/88UG

ISTITUTO ZOOPROFILATTICO SPERIMENTALE
DELLE VENEZIE
AGGREGATO ALLA
UNIVERSITÀ DI PADOVA

DIRETTORE: PROF. DOTT. G. GAGLIARDI

CONTROLLO ALIMENTI

CERTIFICATO N. 6194-99

data 30/5/88

6 Campione di Mangime

della Ditta A. Rieper - Vandoies (BZ)

prelevato il 17/5/88 presso la stessa

inviato da Dr. Tauber - Via Raas, 6 - Sciaves (BZ) 39040

il 20/5/88 con lettera /

DESCRIZIONE DEL CAMPIONE - RISULTATO DEL CONTROLLO - GIUDIZIO							
Campione in unico esemplare sigillato.							
Ricerca biologica di attività estrogena:							
6194 - Camp.	92	Altromin	R	Lotto	8806		NEGATIVA
6195 - "	93	"	MT	"	"		"
6196 - "	94	"	H	"	"		"
6197 - "	95	"	A	"	"		"
6198 - "	96	"	MSK	"	"		"
6199 - "	97	"	CL	"	"		"

IL LABORATORISTA
Dr. GIULIANO BERSANI

ISTITUTO ZOOPROFILATTICO SPERIMENTALE delle VENEZIE PADOVA

IL DIRETTORE
Prof. Dott. G. Gagliardi

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)

MT/330

UNIVERSITÀ DEGLI STUDI DI MILANO
ISTITUTO DI ISPEZIONE DEGLI ALIMENTI
DI ORIGINE ANIMALE "Pietro Stazzi"
CATTEDRA I

VIA CELORIA, 10 - TEL. 2361528
20133 MILANO

Codice Fiscale n. 80121280150

Spett.le
A. Rieper
Industria Mangimi
I-39030 Vandoies

Milano, li 20.5.88

OGGETTO: analisi nitrosammine di n.° 1 campione di campione n. 99
Altamin Rieper tipo MT prelevato in loco
lotto 8806 in data 17.5.88

ANALISI MICROBIOLOGICA

Conta totale

Indicatori fecali:
Strept. D. :
E. Coli

Germi patogeni:
Stafilococchi:
Salmonelle :
Clostridi :
Lieviti :
Muffe :

ANALISI CHIMICO-FISICHE

nitrosammine : assenti

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NENZIATI)

GIUDIZIO:

MT 1R&C -

altromin international

Scientific department

ALTROMIN GmbH
D-4937 Lage, Lippe
Lange Straße 42 · Postfach 1120
Tel. (05232) 63013, Ext. 20
Telex 931423

Analytical Report of sample:

Definition: Altromin Rieper Muster81 — Type: MT

Batch/Lotto No.: 8806

Chemical analysis (Referring to dry matter)	%
Crude protein	23,15
Crude fat	5,39
Crude fiber	4,45
Ash	6,10
Calcium	0,97
Phosphorus	0,79

Moisture	10,2
Hardness check: Pellet hardness in kg/cm² acc. Kahl	19
Sense evaluation: Smell	ok
Appearance	ok

Vitamin A	20.100 UI/kg
Vitamin E	105 mg/kg

260583
Date

LIFE SCIENCE RESEARCH ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)
Signature

APPENDIX IV

CERTIFICATE OF ANALYSIS

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALBERTO MANZIATA)

LABORATORI DI CONTROLLO

Richiesta N. ....

del 29-04-88

per il Laboratorio ☐ Ric. e Svil. ☒ C. Chimico ☐ C. Biologico

Prodotto ESATOSINA SOL. Codice N. 60-6062 Lotto N. 15/393 B

Fornitore/Rep. FARMITALIA Carlo Erba N. di Lotto del fornitore 80100

Firma del richiedente A. Buniva

Campionato il 2/5/88 da Diana

Secondo il P.S.O. N. AH0.46

Campionati N. 22 da 100cc di contenitori su 1087 del 4/5/88

Aspetto dei contenitori VS-20

CONTROLLO CHIMICO

Analisi N. CA7 048-3 P.S.O. N. AF01400 Data 4/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (.....°C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso t=			Cromatografia		
Peso medio Grammi	5,05	A.M.	Titolo		
Carat. spettrofot.					
Ceneri			SOLUBILITA'	BUONA	A.I
Arsenico					
Metalli pesanti			FdPNa$_3$H gr./f.l.	4,43	A.I
Ferro					
Cloruri			UNIFORMITA' PESO	Conforme	A.I
Solfati					
Acido ossalico			CONTROLLO PARTICELLE	NEI LIMITI	A.I.
Ammoniaca					
Calcio					

Approvato ☒ Respinto ☐ 25/5/88 Responsabile Laboratorio

CONTROLLO BIOLOGICO

Data ....

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità			
Apirogenicità			
Atossicità			
Titolo microbiologico			

LIFE SCIENCE RESEARCH ROMA TOXICOLOGY CENTRE S.p.A. (S. ANNUNZIATA)

Dr. A...

Responsabile Laboratorio

DATI DI PRODUZIONE

Tipo di soluzione infusionale: ESAFOSFINA gr 5 sem/l.

N° lotto interno : P8/393 B - liofilizzato 80/100 B.F.

Data di preparazione : 23/04/88

DATI DI LABORATORIO C.Q.

Controllo visuale:

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}$/g o $\bar{m}$
50 um-100 um								
100 um-300 um								
>300 um								

Controllo microscopico:

Ø PARTICELLE	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}$/g o
<10 µm								
10 um - 25 µm								
25 um - 50 µm								
>50 µm								

25/5/88

Controllo particellare:

volume campionato: 5ml

Ø PARTICELLE (µm)	BIANCO	I° CAMP.	II° CAMP.	III° CAMP.	IV° CAMP.	V° CAMP.	$\bar{m}$	$\bar{m}$/g
2	32	714	764	722	417	470	585,4	23
5	4	144	157	164	116	117	135,6	5
10	1	17	17	24	22	26	20,2	8
20	0	0	1	0	1	1	0,6	2
25	0	0	0	0	0	0	0	
50	0	0	0	0	0	0	0	

LABORATORI DI CONTROLLO

Richiesta N. ..... del 29-04-88

per il Laboratorio ☐ Ric. e Svil. ☑ C. Chimico ☒ C. Biologico

Prodotto ESAFOSFINA SOL Codice N. 60.6062 Lotto N. 25/3933 del 29-4-

Fornitore/Rep. FAL POWER N. di Lotto del fornitore 80100 P.F.

Firma del richiedente A. Buiese

Campionato il 2/5/88 da Dare

Secondo il P.S.O. N. AM046

Campionati N. 24 da 100cc di contenitori su 10687 da 4782AJF

Aspetto dei contenitori VETRO

CONTROLLO CHIMICO

Analisi N. 30/061/02 P.S.O. N. 30/010 Data 24/5/88

SAGGI	RISULTATI	SIGLA	SAGGI	RISULTATI	SIGLA
Descrizione			Sost. ossidabili		
Identità			Solidi totali		
Int. fus. o di ebol.			Fosforo inorganico		
Potere rotatorio			Disgregazione		
Peso spec. (.....°C)			Conten. in volume		
Ind. di rifrazione			Colore della sol.		
pH			Nitriti		
Acqua (K.F.)			Nitrati		
Perd. peso t =			Cromatografia		
Peso medio			Titolo		
Carat. spettrofot.					
Ceneri					
Arsenico					
Metalli pesanti					
Ferro					
Cloruri					
Solfati					
Acido ossalico					
Ammoniaca					
Calcio					

Approvato ☐ Respinto ☐

Responsabile Laboratorio

CONTROLLO BIOLOGICO

Data 24/5/88

SAGGI	RISULTATI	ANALISI N.	SIGLA
Sterilità	STERILE	BS/069/04	MP
Apirogenicità	Apirogeno	BP2/084/07	
	ATOSSICO	BT2/098/23	
Atossicità			
Titolo microbiologico			

LIFE SCIENCE RESEARCH
ROMA TOXICOLOGY CENTRE S.p.A.
(Dr. ALFREDO NUNZIATA)